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Effects of Inhalation of 0.12 and 0.25 Parts per Million Ozone on the Proximal Alveolar Region of Juvenile and Adult Rats

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16. ABSTRACT

One day old or 6 week old male rats were exposed to either 0.25 ppm O_3 12 hr/day or to continuous room air for 6 weeks. An additional group of 6 week old rats was exposed to 0.12 ppm of O_3 for the same time period. In the animals exposed to 0.25 ppm O_3 from 1 day of age (juvenile animals), the number of type I epithelial cells doubled, their mean surface area decreased 38%, and their mean thickness increased 24%. The number of alveolar macrophages doubled. Adult animals exposed to 0.25 and 0.12 ppm O_3 showed similar patterns of changes in the epithelium of the proximal alveolar region. Compared to the juvenile animals, the adult 0.25 ppm O_3 exposed animals showed more reaction in the interstitium with a doubling of interstitial macrophages, suggesting a mild inflammatory stimulus in the interstitium. Animals exposed to 0.12 ppm O_3 showed smaller, but statistically significant changes in the alveolar type I epithelium, suggesting a relatively linear concentration response relationship. These results suggest that low concentrations of O_3 cause a chronic epithelial injury in the proximal alveolar region of both juvenile and adult rats and that the extent of these changes occurs in a concentration-dependent manner, even at concentrations at low as 0.12 ppm.

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Effects of Inhalation of 0.12 and 0.25 Parts per Million Ozone on the Proximal Alveolar Region of Juvenile and Adult Rats

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The degree of lung injury caused by prolonged inhalation of low levels of ozone (O_3) is of interest since urban environmental levels periodically reach 0.2 to 0.3 ppm. Since the area of the junction of the conductive and respiratory regions of the lung has been reported as the major site of injury due to O_3 inhalation, techniques were devised to specifically study alveolar tissue from this region. One-day-old or 6-week-old male rats were exposed to either 0.25 ppm O_3 12 hours/day or to continuous room air for 6 weeks. An additional group of 6-week-old rats were exposed to 0.12 ppm O_3 for the same time period. All lungs were fixed at the end of the exposure by intratracheal installation of buffered 2% glutaraldehyde. Cylinders of tissue containing a cross-section of a terminal bronchiole were punched out of lung tissue slices using a sharpened cannula. These tissue cylinders were oriented, embedded in Epon, serial sectioned until the first alveolar duct bifurcation was reached, and then thin sectioned for electron microscopy. Qualitative examination of the tissue revealed little observable damage to the proximal alveolar tissues. However, by ultrastructure morphometric analysis, significant changes occurred in the alveolar epithelium of the proximal alveolar region of all exposed animals. In the animals exposed to 0.25 ppm O_3 from 1 day of age (juvenile animals), the number of type 1 epithelial cells doubled, their mean surface area decreased 38%, and their mean thickness increased 24%. The number of alveolar type 2 epithelial cells increased, and the number of alveolar macrophages doubled. Adult animals exposed to 0.25 ppm O_3 showed similar patterns of changes in the epithelium of the proximal alveolar region and in addition had a doubling of interstitial macrophages, indicating a mild inflammatory stimulus in the interstitium. Adult animals exposed to 0.12 ppm O_3 showed smaller, but statistically significant changes in the alveolar type 1 epithelium, suggesting a relatively linear concentration-response relationship. The change in number and size of type 1 cells in all exposed animals is consistent with an increased cell turnover rate due to prolonged O_3 inhalation. These results suggest that low concentrations of O_3 cause a chronic epithelial injury in the proximal alveolar region and that the extent of these changes occurs in a concentration-dependent manner, even at concentrations as low as 0.12 ppm. No statistically significant age-dependent effects were found.

Additional key words: Lung, Morphometry, Stereology, Electron microscopy, Oxidant lung injury.

The current US National Ambient Air Quality Standard for ozone (O_3) is 0.12 ppm. This level may be exceeded in some major metropolitan areas, especially during the summer months (24). Considerable debate still exists as to whether or not 0.12 ppm O_3 is a level at which no significant injury to lung structure will occur from either acute or chronic exposures. The alveoli in the proximal portions of the acinus have been recognized as a primary site of lung injury following inhalation of O_3 since an early study by Stokinger, Wagner, and Dobrogorski (30). This area of the lung may be termed the proximal alveolar region and has been the focus of a large number of recent studies (3, 9, 11–14, 22, 26–28). In general, alterations in the proximal alveolar region caused by concentrations less than 0.5 ppm O_3 have been reported as minimal to slight.

Plopper *et al.* (26) reported that 7 days of continuous exposure to 0.1 ppm O_3 caused subtle qualitative changes in the proximal alveolar region of some exposed rats, including thickening of type 1 epithelial cells and an increase in the numbers of alveolar macrophages. Similar changes were reported by Schwartz *et al.* (27) after 7 days of exposure to 0.2 ppm O_3. Following 90 days of exposure to 0.2 ppm O_3, Boorman, Schwartz, and Dungworth (3) reported that there were slight increases in the arithmetic mean thickness of the air-blood barrier and

in the volume density of the epithelium. The proximal portions of the respiratory acinus represent relatively small, oriented regions of lung parenchyma that cannot be studied by random selection of tissue samples. In the current study methods were developed to reproducibly isolate oriented cross-sections of the proximal alveolar region that could be used to quantitate changes occuring in cell populations, average cell characteristics, and the functional characteristics of the alveolar septa.

An important issue is whether or not there are populations of individuals who are more sensitive to long-term exposures to low doses of O_3. The young, the old, and individuals with chronic lung diseases have all been postulated to be more susceptible to lung injury caused by exposure to low levels of O_3. Since the rat is born with an immature lung and completes the process of alveolarization during the first 3 weeks of life, this animal provides a good model to examine the effects of O_3 on the developing lung. The present study was conducted to address the questions of whether or not changes in lung structure are caused by exposure to low levels of O_3 and whether the degree of maturity of the lung at the beginning of the exposure is a critical factor which affects the extent of injury.

MATERIALS AND METHODS

Animals and Exposure Conditions

All rats were maintained in 0.323 m^3 stainless-steel Rochester-type chambers during exposures to filtered air or O_3. Ozone was generated from 100% oxygen (extra-dry) with an Orec model 03V5-0 Ozonizer (Ozone Research and Equipment Corporation, Phoenix, Arizona), mixed with dry, filtered air, and directed into the top of the chamber. Flow within the chambers was 323 liters/minute. The concentration of O_3 in the chambers was continuously monitored with a chemiluminescence O_3 analyzer (Bendix Corporation, Ronceverte, West Virginia) which was calibrated twice a month by a gas-phase titration technique (32).

Male Fischer 344 rats (CDF, F-334/Crl BR, Charles River Laboratories, Wilmington, Massachusetts) which were either 1 day old or 6 weeks old at the beginning of the exposure were used in these experiments. For brevity, the former group will be termed juvenile rats and the latter adult rats. Both groups were exposed to 0.25 ppm O_3 for 12 hours/day, 7 days/week for 6 weeks. The standard deviations (SD) of the exposure concentrations were 0.0082 and 0.0055 for the juvenile and adult groups, respectively. Another group of adult rats was exposed to 0.12 ± 0.0047 ppm O_3 for the same time period. All O_3-exposed rats were kept continuously in the chambers and exposed to filtered air only for the remaining 12 hours of each day. Control rats were maintained continuously in identical chambers and exposed only to filtered air. Adult rats were weight matched at the beginning of the exposure. To begin the exposure of the juvenile rats at 1 day of age, timed pregnant female rats were placed in the chambers 1 week prior to delivery and exposed to filtered air. Ribboned sheets of Teflon were used as nesting material since this material would not react with O_3. Preliminary tests with wood chips or shredded, ink-free newspaper as bedding showed that these materials reacted with O_3. These reactions decreased the concentra-

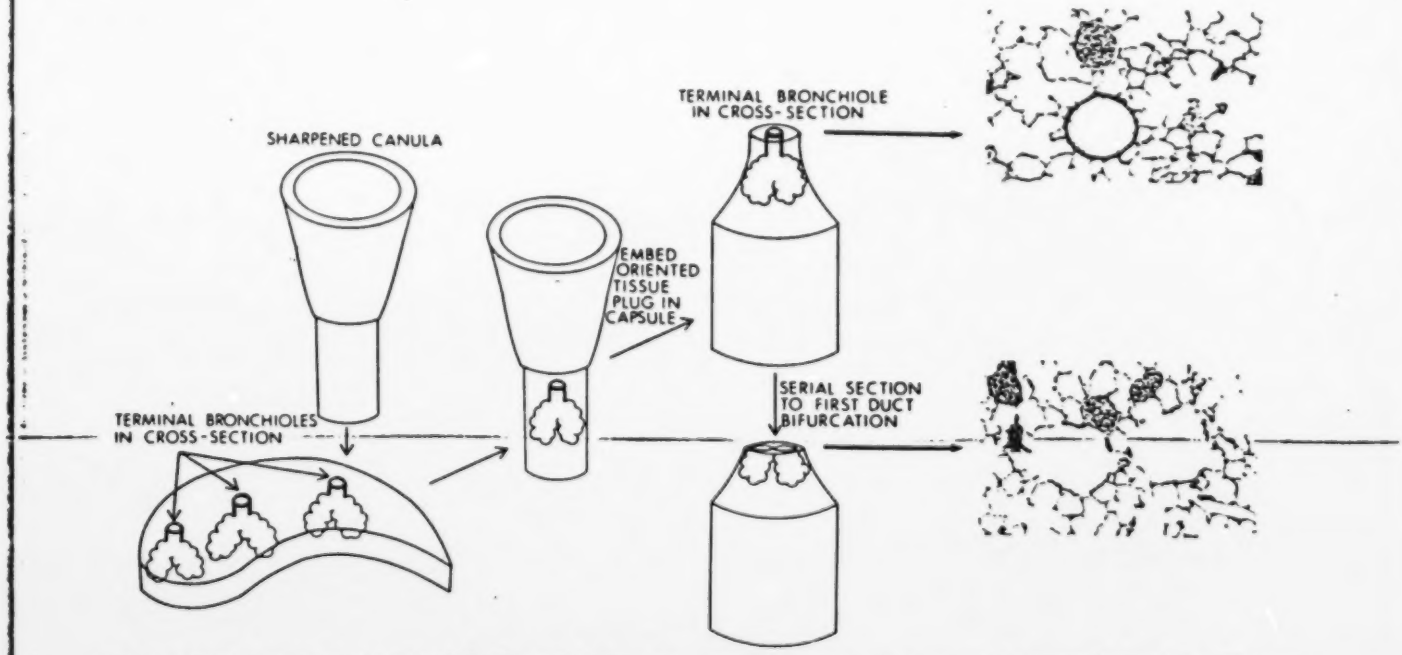

FIG. 1. Method of isolation of proximal alveolar regions from rat lung tissue. A lung lobe is cut into a series of slices and they are examined with a dissecting microscope. Cross-sections of terminal bronchioles are punched out with a sharpened cannula and the tissue is oriented in the capsule in the last stage of embedding.

tion of O_3 within the bedding compared to that measured at the chamber intake port. Similar tests with ribboned Teflon showed that this material did not cause alterations in O_3 concentrations. Within 24 hours of birth, litters were reduced to six male pups and the exposure begun. If litters did not contain six male pups, male pups from other litters were added to achieve a complement of six. Mothers were continuously kept in the cages with the pups for 3 weeks. At the end of this period, which is the normal time of weaning, mothers were removed from the cages. Rats received food and water *ad libitum*. Maintenance of the animals was conducted for 1 hour each morning prior to the start of the daily O_3 exposure period.

Histologic and Morphometric Methods

Each rat was anesthetized with intraperitoneal pentobarbital and its trachea cannulated. The abdominal cavity was opened, both hemidiaphragms were punctured, and 2% glutaraldehyde in 0.084 M Na cacodylate buffer was instilled into the lungs at a constant fixative pressure of 20 cm of H_2O above the chest wall. To isolate the alveolar region adjacent to terminal bronchioles, the left lung was transversely cut into a series of slices which were examined with a dissecting microscope (Fig. 1). Circular cross-sections of terminal bronchioles were identified and tissue cylinders containing them were punched out with a sharpened 20-gauge Becton Dickenson (Rutherford, New Jersey) Luer stub cannula. Terminal bronchioles from all slices of the lobe were included in the samples. The tissue cylinders were washed in cacodylate buffer, postfixed in osmium tetroxide, and *en bloc* stained with 2% uranyl acetate. The tissue was then dehydrated in a series of graded alcohols and propylene oxide, oriented, and embedded in Epon 812. Using a Sorvall MT 5000 ultramicrotome, all blocks were serially sectioned through the terminal bronchiole and only those blocks in which a first alveolar duct bifurcation was reached were selected for study. The area surrounding the first alveolar duct bifurcation was defined as the proximal alveolar region. Excess tissue was trimmed

TABLE 1. ABSOLUTE VOLUMES, SURFACE AREAS, AND DIFFUSION CAPACITY FOR PROXIMAL ALVEOLAR REGION OF THE LUNG[a]

	Juvenile rats		Adult rats			Overall contrasts[b]		
	Controls	0.25 ppm O_3	Controls	0.12 ppm O_3	0.25 ppm O_3	Gas	Age	Gas × age
n	8	8	12	8	8			
Body weight (gm)	130 ± 5	129 ± 2	267 ± 2	262 ± 3	249 ± 8[c]	−	++	−
Total lung volume (cm^3)	5.39 ± 0.17	5.40 ± 0.14	8.12 ± 0.22	8.20 ± 0.17	7.97 ± 0.26	−	++	−
Basement membrane surface density (cm^2/cm^3)	439 ± 15	434 ± 23	493 ± 14	479 ± 15	514 ± 27	−	++	−
Volumes ($V/S_{EP}BM$) (μm)								
Air	20.511 ± 0.799	21.005 ± 1.246	18.156 ± 0.569	18.769 ± 0.581	17.424 ± 1.073	−	++	−
Capillary lumen	1.108 ± 0.061	1.016 ± 0.063	1.315 ± 0.057	1.193 ± 0.160	1.239 ± 0.076	−	++	−
Tissue	1.320 ± 0.034	1.508 ± 0.065[c]	0.996 ± 0.025	1.075 ± 0.038	1.174 ± 0.032[c]	++	++	−
Type 1 epithelium	0.241 ± 0.008	0.296 ± 0.011[c]	0.197 ± 0.005	0.218 ± 0.007[c]	0.238 ± 0.007[c,d]	++	++	−
Type 2 epithelium	0.097 ± 0.009	0.127 ± 0.007[c]	0.074 ± 0.005	0.071 ± 0.008	0.099 ± 0.010[c,d]	++	++	−
Cellular interstitium	0.316 ± 0.016	0.317 ± 0.022	0.195 ± 0.008	0.203 ± 0.012	0.226 ± 0.007[c]	−	++	−
Noncellular interstitium	0.260 ± 0.008	0.289 ± 0.013	0.264 ± 0.007	0.268 ± 0.016	0.291 ± 0.015	+	−	−
Endothelium	0.367 ± 0.008	0.381 ± 0.019	0.250 ± 0.007	0.284 ± 0.009[c]	0.273 ± 0.007	−	++	−
Macrophages	0.039 ± 0.004	0.099 ± 0.018[c]	0.019 ± 0.002	0.032 ± 0.008	0.047 ± 0.007[c]	++	++	±
Surface area (SA)[e]								
% type 1	96.2 ± 0.3	95.1 ± 0.2[c]	98.1 ± 0.2	98.8 ± 0.2[c]	97.6 ± 0.2	++	++	−
% type 2	3.8 ± 0.3	4.9 ± 0.2[c]	1.9 ± 0.2	1.2 ± 0.2	2.4 ± 0.2[d]	++	++	−
Capillary endothelium	101.3 ± 1.7	100.5 ± 1.9	96.6 ± 0.2	98.3 ± 2.3	97.9 ± 0.2	−	+	−
D_T ($\mu l\ O_2 \cdot min^{-1} \cdot mm\ Hg^{-1} \cdot cm^{-2}$)	0.811 ± 0.043	0.707 ± 0.013[c]	0.876 ± 0.024	0.883 ± 0.027	0.770 ± 0.023[c,d]	++	+	−
Tissue thickness								
Harmonic mean (μm)								
Air to plasma	0.415 ± 0.016	0.469 ± 0.010[c]	0.373 ± 0.009	0.373 ± 0.009	0.426 ± 0.009[c,d]	++	++	−
Arithmetic mean								
Epithelium	0.338 ± 0.012	0.423 ± 0.017[c]	0.267 ± 0.081	0.288 ± 0.008	0.337 ± 0.012[c,d]	++	++	−
Type 1	0.250 ± 0.008	0.311 ± 0.012[c]	0.197 ± 0.005	0.220 ± 0.007[c]	0.244 ± 0.007[c,d]	−	++	−
Type 2	2.613 ± 0.207	2.613 ± 0.131	4.416 ± 0.372	5.682 ± 0.417[c]	4.263 ± 0.365	+	++	−
Interstitium	0.572 ± 0.018	0.604 ± 0.032	0.467 ± 0.015	0.474 ± 0.021	0.522 ± 0.015[c]	−	++	−
Endothelium	0.363 ± 0.011	0.378 ± 0.015	0.260 ± 0.008	0.290 ± 0.011[c]	0.279 ± 0.009	−	++	−

[a] All data are mean ± SE. For the adult animals, comparisons among three groups were done using a multiple comparison procedure (8).

[b] Overall contrasts for significant effects due to age and/or to exposure to O_3 include only those groups exposed to air or 0.25 ppm O_3: $++p < 0.01$; $+p < 0.05$; $\pm 0.05 < p < 0.10$; $-p > 0.10$ for two-sided tests.

[c] $p < 0.05$ for comparison to their respective control group.

[d] $p < 0.05$ for comparison to animals exposed to 0.12 ppm O_3.

[e] Type 1 SA is the SA of basement membrane under type 1 cells; type 2 SA is the air surface of type 2 cells excluding the extra SA contributed by microvilli; endothelial SA is the luminal surface of the endothelial cells expressed as a percentage of the alveolar basement membrane SA.

TABLE 2. MORPHOMETRICALLY DETERMINED CHARACTERISTICS OF CELLS IN THE PROXIMAL ALVEOLAR REGION OF THE LUNG[a]

	Juvenile rats		Adult rats			Overall contrasts[b]		
	Control	0.25 ppm O_3	Control	0.12 ppm O_3	0.25 ppm O_3	Gas	Age	Gas × age
n	8	8	12	8	8			
Total no. of cells (/mm²)	1987 ± 112	2290 ± 110	1611 ± 58	1756 ± 80	1778 ± 101	+	++	−
Alveolar type 1 cells								
No. (/mm²)	126 ± 12	220 ± 34[c]	103 ± 6	140 ± 15[c]	185 ± 15[c,d]	++	−	−
Average volume (μm³)	2040 ± 199	1551 ± 203	1947 ± 144	1730 ± 266	1374 ± 158[c]	++	−	−
Average surface area (SA) (μm²)	8679 ± 882	5342 ± 795[c]	10297 ± 717	7928 ± 948[c]	5744 ± 556[c]	++	−	−
Average basement membrane SA/cell (μm²)	8189 ± 818	5088 ± 770[c]	9910 ± 720	7690 ± 834[c]	4449 ± 507[c]	++	−	−
% total lung cells	6	10	6	8	10			
Alveolar type 2 cells								
No. (/mm²)	224 ± 18	300 ± 20[c]	171 ± 12	212 ± 26	238 ± 26[c]	++	++	−
Average volume (μm³)	446 ± 41	431 ± 25	447 ± 33	357 ± 52	420 ± 22	−	−	−
Average SA (μm²)								
With microvilli	1052 ± 63	965 ± 36	209 ± 29	154 ± 21	131 ± 25	−	+	−
Without microvilli	172 ± 11	166 ± 10	109 ± 12	64 ± 9[c]	105 ± 12[d]	−	++	−
% total lung cells	11	13	11	12	13			
Endothelial cells								
No. (/mm²)	983 ± 54	1051 ± 39	885 ± 24	943 ± 37	855 ± 43	−	++	±
Average volume (μm³)	379 ± 18	364 ± 18	284 ± 8	303 ± 10	322 ± 12[c]	−	++	−
Average SA (μm²)	228 ± 28	243 ± 22	1101 ± 34	1057 ± 55	1160 ± 49	−	+	±
% total lung cells	50	46	55	54	48			
Interstitial cells								
No. (/mm²)	611 ± 46	635 ± 51	426 ± 33	433 ± 18	454 ± 35	−	++	−
Average volume (μm³)	530 ± 31	512 ± 42	488 ± 40	469 ± 22	520 ± 44	−	−	−
% total lung cells	31	28	26	25	26			
Macrophages								
No. (/mm²)	43 ± 7	85 ± 21	25 ± 3	27 ± 5	46 ± 6[c,d]	++	++	−
Average volume (μm³)	1230 ± 374	1785 ± 707	945 ± 197	1209 ± 252	1009 ± 98	−	−	−
% total lung cells	2	4	2	2	3			

[a] All data are mean ± SE. For the adult animals, comparisons among three groups were done using a multiple comparison procedure (8).

[b] Overall contrasts for significant effects due to age and/or to exposure to O_3 did not include the group exposed to 0.12 ppm O_3: $++p < 0.01$; $+p < 0.05$; $\pm 0.05 < p < 0.10$; $-p > 0.10$ for two-sided tests.

[c] $p < 0.05$ for comparison to controls.

[d] $p < 0.05$ for comparison to animals exposed to 0.12 ppm O_3.

away, and thin sections containing the bifurcation and the surrounding alveoli were picked up on 200 mesh grids. These sections usually covered 10 to 15 grid squares.

Electron micrographs were taken in each of the four corners of the grid squares and enlarged to ×8500 on 11 × 14 inch photographic paper. A counting overlay with 112 lines, each 2 cm long, was placed over each micrograph; point and intercept counts were tallied to determine volume and surface densities. The number of nuclear profiles of each cell type found on 20 consecutive complete grid spaces (N_A) was counted directly on the electron microscope viewing screen. Gunderson's unbiased counting rule (15) was used except for the limitation that the extension of nuclei beyond the edges of the grid space could not be visualized. The area of these grid spaces was determined by photographing one grid space from each grid at ×500 and digitizing its area. The numerical densities (N_V) of alveolar cells in this region were calculated according to the formula $N_V = N_A/\bar{D}$. The values for mean nuclear diameters, $\bar{D}$, for the alveolar cells for control and O_3-exposed rats are from the data for control Fischer 344 rats reported by Pinkerton *et al.* (25). Previous studies of lung oxidant injury have shown that low levels of tissue injury are not associated with changes in the $\bar{D}$ of cells (17) and that even the severe tissue injury caused by exposure to 85% oxygen resulted in changes of 11% or less in the $\bar{D}$ for all alveolar cells except type II cells (7). Since the concentration of O_3 used in the present study does not cause extensive injury to alveolar tisue, it was assumed that little change would occur in the $\bar{D}$ values for lung cells and that the values for normal animals could be used for exposed animals.

Standard morphometric formulas were used to calculate volume, surface, and numerical densities of cells and other tissue components in the alveolar region (7, 35). If these parameters are expressed only as densities, differences in the degree of lung inflation between groups could lead to incorrect interpretations of the results. To solve this problem, the data were normalized by dividing each morphometrically determined volume, surface, and numerical density by the surface density of alveolar basement membrane in the same sample. Thus, volume, surface, and numerical densities are expressed relative to the surface density of epithelial basement membrane within a standard volume of lung tissue from the proximal alveolar region. Calculations for harmonic and arith-

metic mean thicknesses, the tissue component of diffusion capacity, mean cell volumes, and mean cell surface areas are independent of this normalization factor.

The above normalization reduces variance caused by differences in the degree of inflation of the fixed lungs since epithelial basement membrane surface area will increase or decrease proportionately with different inflation volumes. Bachofen and Weibel (1) first used this technique to express morphometric data from human lungs after acute lung injury. The rationale for this normalization method is that the basement membrane is a collagenous structure which is thought to undergo minimal stretching at physiologic levels of inflation. Hayatdavoudi *et al.* (16) have shown that when normal rat lungs are fixed by rapid intratracheal instillation of glutaraldehyde at 20 cm of final pressure, the level used in the present study, the total lung volume is approximately 70% of total lung capacity as defined by physiologic measurements. Since this is within the highly compliant range of lung expansion, stretching of the basement membrane should not occur and this structure can serve as an internal standard and a stable reference point. In these experiments the normalization factor was the same between the age-matched exposed and control groups.

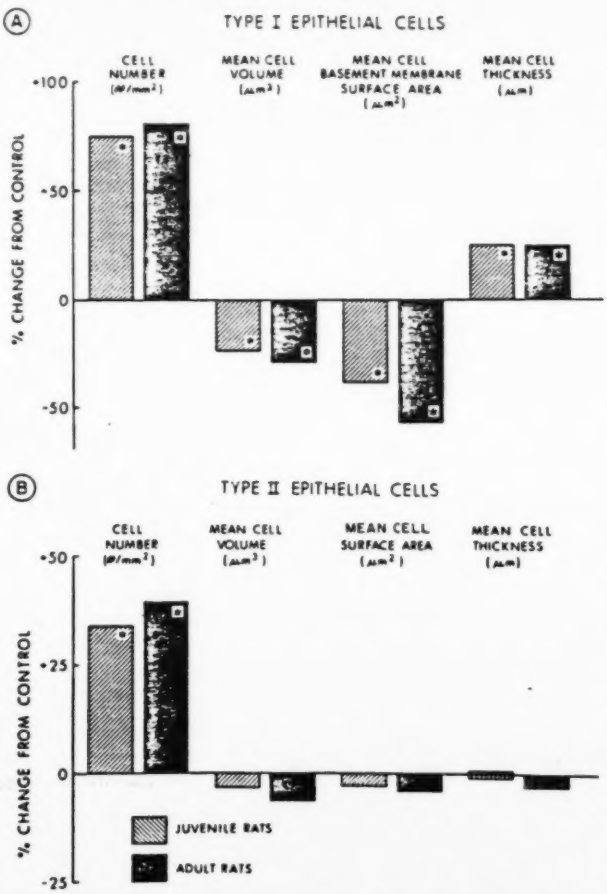

FIG. 2. The epithelium in the proximal alveolar region. The changes in the morphometric characteristics of type 1 and 2 epithelial cells of juvenile and adult rats expressed as percent change from control values following exposure to 0.25 ppm of O_3 for 6 weeks. * $p < 0.05$ for analysis of raw data as reported in Tables 1 and 2.

INTERSTITIAL CELL CHARACTERIZATION

The alveolar interstitial cells in the proximal alveolar region of controls and rats exposed to 0.25 ppm O_3 were categorized according to morphologic criteria to determine which cell types were present. Eight of the 12 controls from the adult animals were randomly selected for this categorization. All the electron micrographs in each series were examined and those interstitial cells which contained at least a portion of a nuclear profile which did not intersect the right or lower margins of the micrograph were included in the differential count.

STATISTICAL ANALYSIS

Responses of the juvenile rats (control and 0.25 ppm O_3) were compared using Student's *t*-tests (two-tailed, with $p < 0.05$ required for statistical significance). Data for the adult rat groups (control, 0.12 ppm, and 0.25 ppm O_3) were analyzed by analysis of variance with subsequent pairwise comparison of group means using Duncan's multiple range test (8). After excluding the 0.12 ppm O_3 adult rat data, one-way analyses of variance on the four remaining groups, along with appropriately selected single degree of freedom contrasts, were used to address the question of possible age susceptibility to O_3 exposure. The overall contrasts reported in Tables 1 and 2 are analogs to a two-way analysis of variance involving gas exposure (air versus O_3), age (juvenile versus adult), and their interaction (gas × age). A positive interaction in the gas × age column of Tables 1 and 2 would mean that the juvenile and adult animals responded differently to the O_3 exposure.

RESULTS

COMPARISON OF EFFECTS OF 0.25 PPM O_3 ON JUVENILE AND ADULT RATS

Substantial effects due to age, independent of exposure, were found for all parameters in Table 1 as indicated by the significant effect of the "age" contrast. These effects primarily reflect the smaller size of the lungs of the young animals. There were some differences for mean lung cell characteristics between the juvenile and adult rats as reflected by the overall contrasts for age shown in Table 2. Neither the juvenile nor the adult animals were found to be more sensitive to the effects of exposure to 0.25 ppm O_3 as shown by the consistently negative contrasts for gas × age in Tables 1 and 2. Because both age groups of animals showed the same response to exposure to O_3, the overall contrasts for the effects of O_3 alone as reported in Tables 1 and 2 under the column "Gas" are interpreted as more powerful than the separate analyses of the data for determining statistically significant responses to 0.25 ppm O_3.

The gain in body weight in the adult rats exposed to O_3 for 6 weeks was 7% less than that of their matched controls ($p < 0.05$), but the final weights for the control

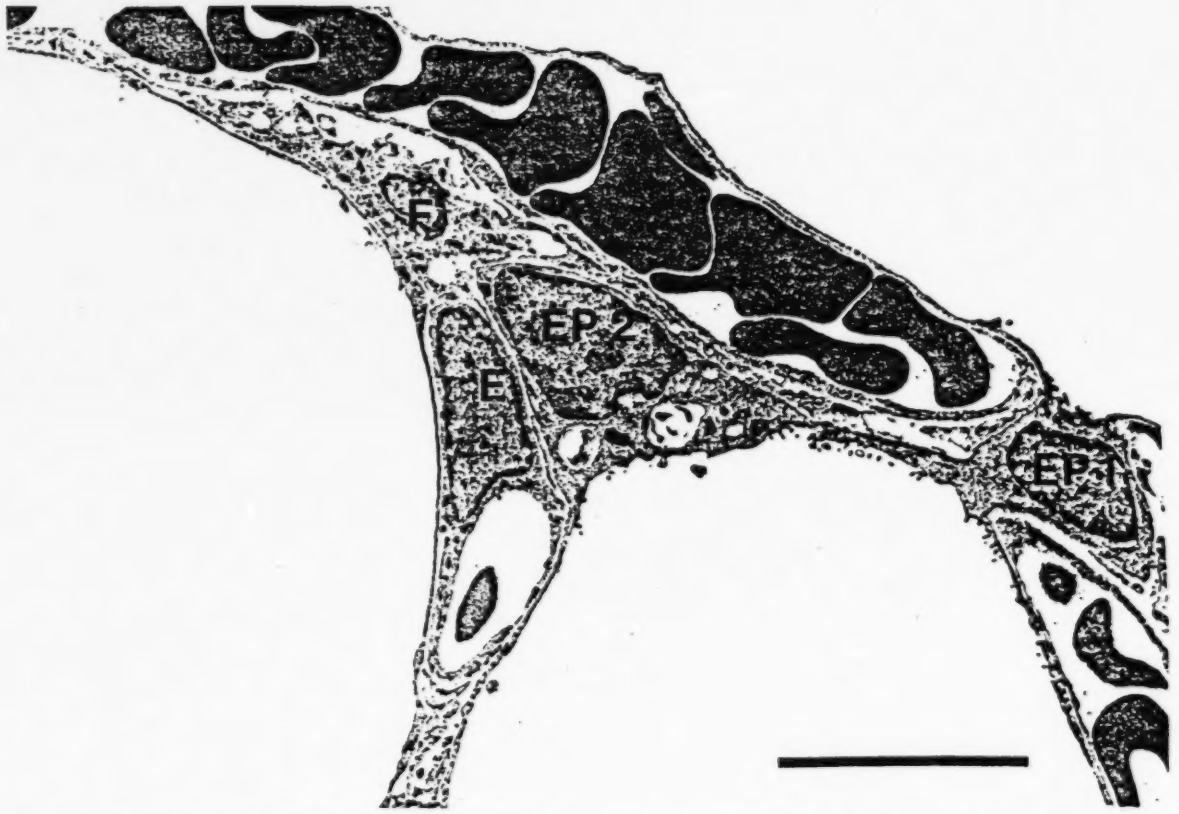

FIG. 3. Alveolar septa from a control juvenile rat. A cuboidal epithelial type 2 cell (*EP2*) and an epithelial type 1 cell (*EP1*) whose cytoplasm is thinly attenuated along the epithelial basement membrane line the alveolar surface. A fibroblast (*F*) in the interstitium and an endothelial cell (*E*) are also present. Bar = 10 μm.

and exposed juvenile rats were virtually identical. Reduction in weight gain by adult rats following exposure to 1 ppm or less of O_3 has been reported (27, 30). The basement membrane surface density was 14% greater in adult rats than in juvenile rats ($p < 0.01$), which may be due to some continued development of the lung during the second 6 weeks of life. There was no significant difference in this parameter between control and O_3-exposed rats for either age group (Table 1).

The volume of tissue in the proximal alveolar region was significantly increased after 6 weeks of exposure to 0.25 ppm O_3 (Table 1). This increase in total tissue volume was attributable to increases in type 1 epithelium, type 2 epithelium, interstitial matrix, and alveolar macrophages.

Changes in the Alveolar Epithelium. Juvenile and adult rats showed similar responses in the alveolar epithelium after exposure to O_3. The number of type 1 cells nearly doubled in both groups and an average type 1 cell was about 25% smaller in volume, covered 40 to 50% less basement membrane surface area, and was 24% thicker than were type 1 cells in respective control animals (Fig. 2*A*). The increased number and the thickened appearance of some type 1 epithelial cells is illustrated by comparing Figures 3 and 4. Although the majority of the epithelium qualitatively appeared normal, there was evidence of type 1 cell injury. Areas of denuded basement membrane were occasionally seen and the plasma membranes of some type 1 cells were ruptured and/or vesiculated. Areas of increased electron lucency were sometimes found in the cytoplasm of these cells.

Adult rats had fewer type 2 cells per unit surface area of basement membrane than did the juvenile rats. In both groups the total number of type 2 cells increased by 25 to 40% (Fig. 2*B*) after exposure to 0.25 ppm O_3, and the fraction of epithelial surface they covered increased slightly (Table 1). Mean cell volume, mean basement membrane surface area, and arithmetic mean thickness of type 2 cells did not statistically vary from that of control animals (Fig. 2*B*). No qualitative changes in the morphology of type 2 cells were noted.

Changes in the Alveolar Interstitium. An increase in the volume of cells in the interstitium was found in adult rats exposed to 0.25 ppm O_3 (Table 1); however, this was not substantiated as a significant overall trend by the contrast analysis. The volume of interstitial matrix increased by 10 to 11% in both juvenile and adult rats exposed to 0.25 ppm O_3. This change in interstitial matrix was statistically significant by analysis of overall contrasts (Table 1). Figure 5 illustrates the changes found in the interstitial compartment.

Differential counts of interstitial cells in the proximal

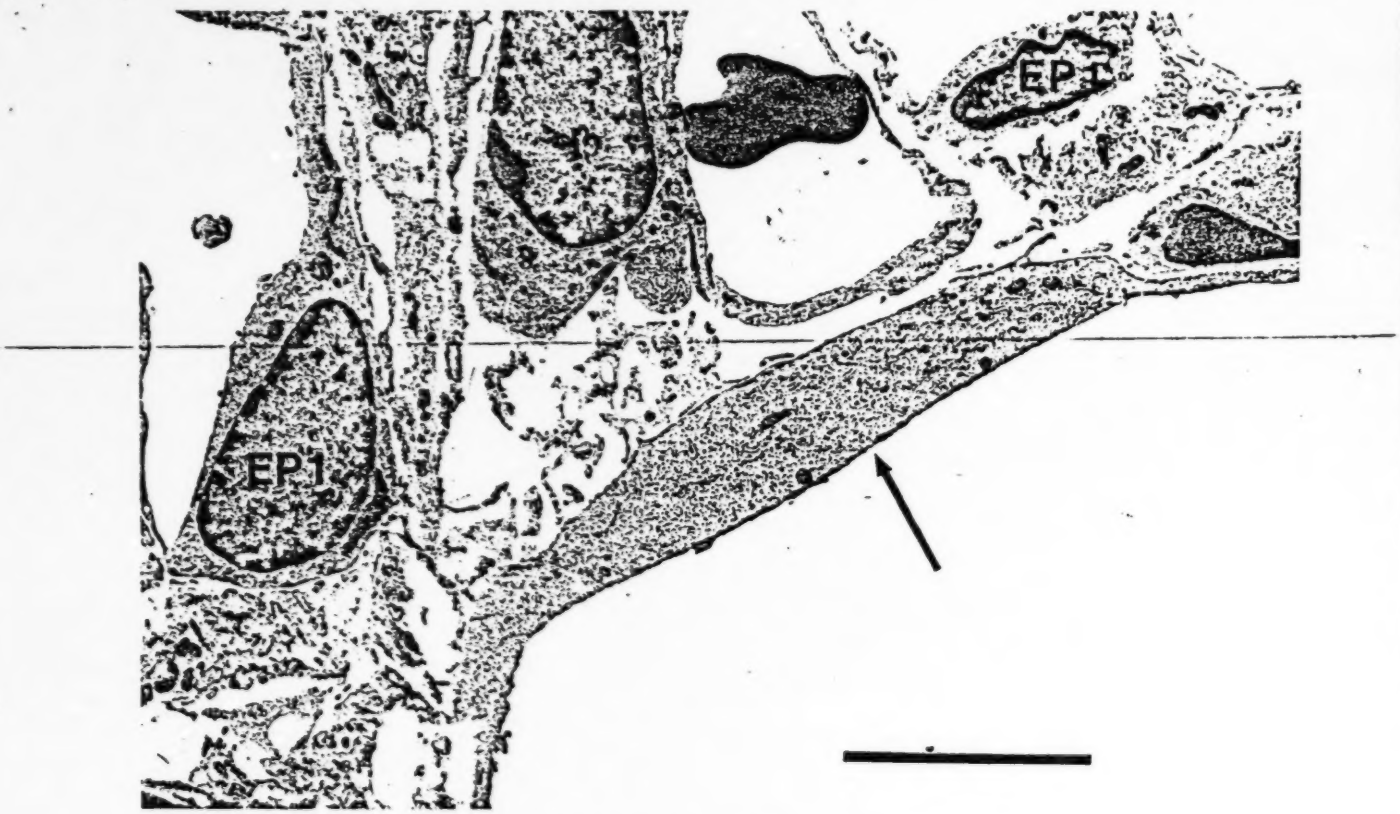

FIG. 4. Alveolar septum from a juvenile rat exposed to 0.25 ppm O_3. An area of thickened type 1 epithelial cell cytoplasm is illustrated (*arrow*). Two profiles of type 1 cell nuclei (*EPI*) are nearby, reflecting the increased population of these cells in the proximal alveolar region following O_3 exposure. Bar = 5 μm.

alveolar region are listed in Table 3. Fibroblasts and interstitial macrophages accounted for approximately 80% of interstitial cells in all groups. The percentage each type of cell contributes to the total was not notably changed in the juvenile rats after O_3 exposure. In the adult rats the percentage of fibroblasts decreased from 70% to 65% and the percentage of interstitial macrophages rose from 10% to 17%.

Changes in Alveolar Macrophages. Alveolar macrophages approximately doubled in both groups of animals exposed to 0.25 ppm O_3 (Table 2) (statistically significant by analysis of overall contrasts). There was no significant change in their mean cell volume. Qualitative examination of these alveolar macrophages showed that their cytoplasm was vacuolated and that they contained numerous debris-laden lysosomes (Fig. 6).

The total numbers of macrophages in the alveolar air spaces and in the alveolar interstitium per unit area of the alveolar basement membrane are shown in Figure 7. The interstitial macrophage data are calculated by using the total numbers of interstitial cells present in each animal (Table 2) and the percent distribution of these cells from Table 3. A statistically significant increase in the total number of interstitial macrophages was found in the young adult animals exposed to 0.25 ppm O_3 for 6 weeks; O_3 increased the number of alveolar macrophages in the air spaces in both age groups of rats.

Effects on Pulmonary Diffusion Capacity. The morphometric estimate of the tissue component of oxygen diffusion capacity in the proximal alveolar region (D_T) was significantly decreased in both the juvenile and young adult rats (Table 1). These values are a component of the calculation of pulmonary diffusion capacity from morphometric parameters as developed by Weibel (33). These decreases, which reflect corresponding significant increases in the harmonic mean tissue thicknesses, indicate that the ultrastructural alterations produced by O_3 increased the functional barrier for gas diffusion across the alveolar septa in the proximal alveolar region.

COMPARISON OF THE EFFECTS OF 0.12 AND 0.25 PPM O_3 ON ADULT RATS

Exposure to 0.12 ppm O_3 caused significant increases in the total volume of type 1 epithelium and endothelium and increases in the arithmetic mean thickness of type 1 and type 2 epithelium compared to control animals. The characteristics of these epithelial cells following

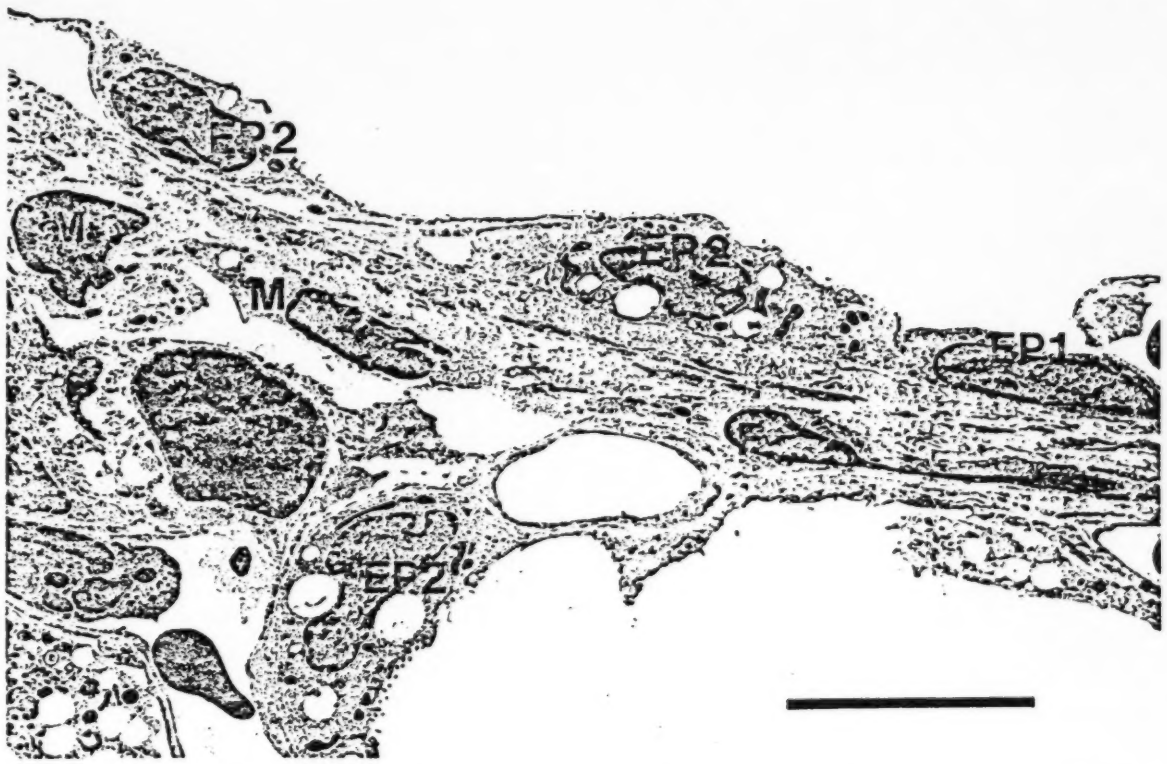

FIG. 5. Alveolar septum from an adult rat exposed to 0.25 ppm O_3. Three type 2 epithelial cells (*EP2*) and a type 1 epithelial cell (*EP1*) line the majority of this alveolar septum. There are more interstitial cells, including fibroblasts (*F*) and macrophages (*M*), and more collagen in the interstitium compared to control rats. Bar = 10 μm.

TABLE 3. EFFECT OF 0.25 PPM O_3 ON DIFFERENTIAL COUNT OF INTERSTITIAL CELLS IN THE PROXIMAL ALVEOLAR REGION[a]

	Percentage of total cell profiles counted						Total cell profiles counted
	Fibroblasts[b]	Macrophages[c]	Pericytes[d]	Lymphocytes[e]	PMN[f]	Indeterminant[g]	
Juvenile rats							
Air	71 ± 4	12 ± 2	6 ± 1	0	0	10 ± 2	262
O_3	70 ± 3	10 ± 1	9 ± 2	0	0	12 ± 3	279
Adult rats							
Air	72 ± 2	9 ± 2	10 ± 1	0	0	10 ± 1	275
O_3	62 ± 5	16 ± 4[h]	7 ± 2	0	0	14 ± 2	358

[a] All data are mean ± SE, $n = 8$.

[b] Fibroblasts were roughly fusiform in shape with elongated processes that extended into the surrounding interstitium. The cytoplasm contained dilated cisternae of rough endoplasmic reticulum. "Contractile interstitial cells" (20) which had bundles of filaments at the cell perimeter were included in this category.

[c] Interstitial macrophages were identified by their round to oval shape and the presence of filopodia on their cell surface. The cytoplasm contained numerous primary and/or secondary lysosomes, free ribosomes and polyribosomes, and little rough endoplasmic reticulum.

[d] Pericytes (34) were enclosed by the capillary basement membrane and had cytoplasmic processes in close apposition to the abluminal side of the endothelial cells.

[e] Lymphocytes were round with a high nucleus to cytoplasm ratio and prominent polyribosomes in the cytoplasm.

[f] Polymorphonuclear leukocytes (PMN) were identified by their lobulated nucleus and its dense chromatin and by the characteristic granules in the cytoplasm.

[g] Indeterminant cells had a high nucleus to cytoplasm ratio and insufficient cytoplasmic markers to properly categorize the cells.

[h] $p < 0.05$ (one-sided test).

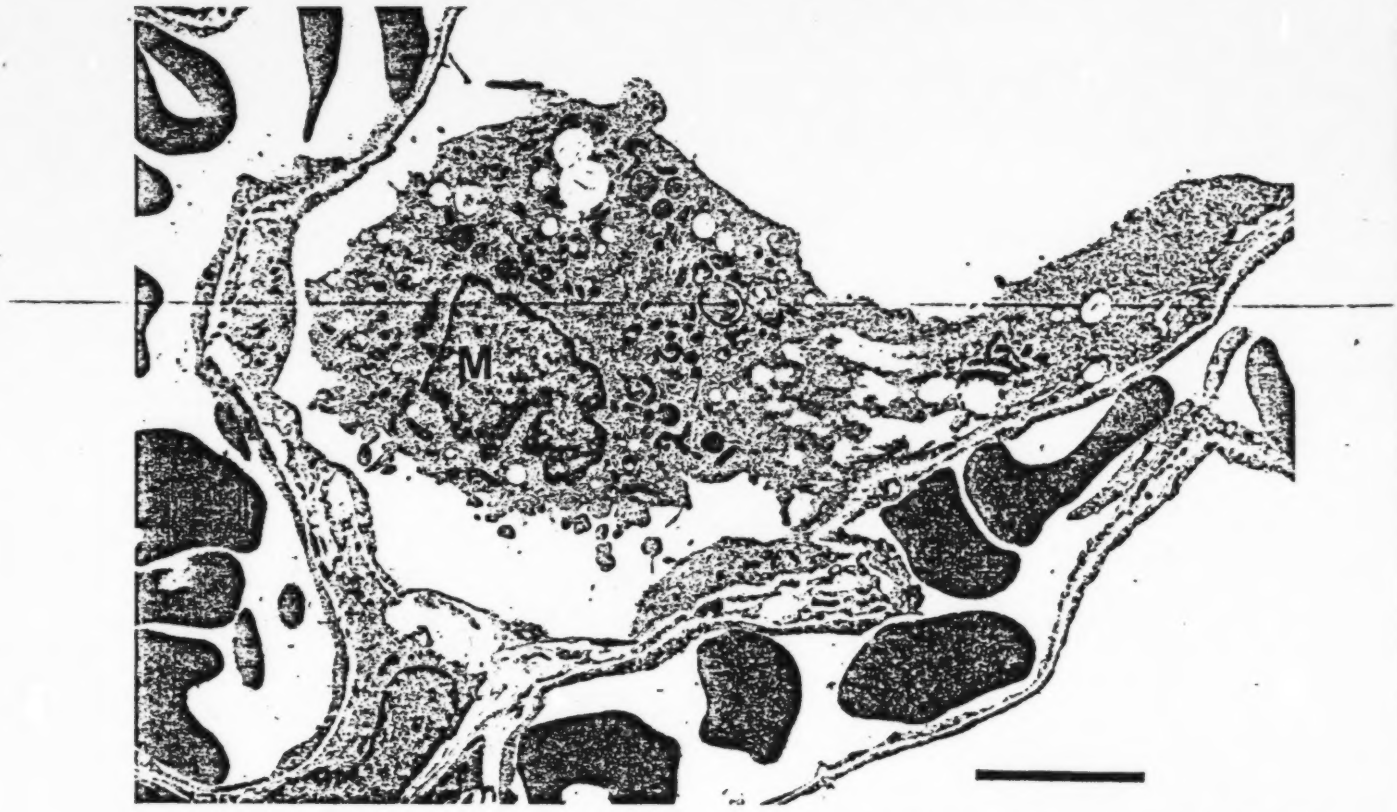

FIG. 6. An alveolar macrophage (*M*) from an adult rat exposed to 0.25 ppm O_3. A large pseudopod extends along the alveolar surface and the cytoplasm contains numerous lysosomes and vacuoles. *Bar* = 5 μm.

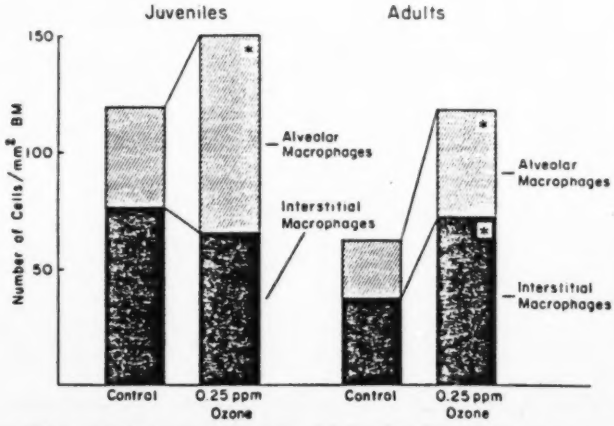

FIG. 7. Number of fibroblasts and interstital macrophages per square millimeter of alveolar epithelial basement membrane in the proximal portions of the ascinus. Fibroblasts are shown in the cross-hatched portion of each *bar*. The numbers of interstitial macrophages are shown in the solid portions of each *bar*. * Significantly different from control, $p < 0.05$.

exposure to 0.12 and 0.25 ppm O_3, compared to control cells, are illustrated in Figure 8. Following exposure to 0.12 ppm O_3, type 1 epithelial cells exhibited the same pattern of structural changes that was found after exposure to 0.25 ppm O_3. These cells were increased in number, covered less basement membrane surface area, and were slightly, but significantly, thicker than type 1 cells from control animals. The number of type 2 epithelial cells increased 24% after exposure to 0.12 ppm O_3, but their mean surface area was less than their mean thickness increased when compared to both control animals and those exposed to 0.25 ppm O_3 (Fig. 8*B* and 9).

The raw data for the volume density of type 1 epithelium per square millimeter basement membrane in the individual proximal alveolar regions from all of the adult animals are illustrated in Figure 10. These data illustrate the biological variability that exists both within and between animals for this lung cell characteristic. It suggests that there is a linear concentration relationship between alterations in the type 1 epithelium and exposure to low concentrations of O_3.

There appears to be no significant threshold for effect of O_3 in causing an increase in the volume of type 1 alveolar epithelium. While there is significant overlap of values found in control animals and values found in exposed animals, it is important to note that 16/48 sites from control animals were below 0.175 while only 3/32 sites from animals exposed to 0.12 ppm O_3 and only 1/32 sites from animals exposed to 0.25 ppm were below this value. At the other end of the scale, only 2/48 sites from control animals were above 0.25 while 8/32 sites

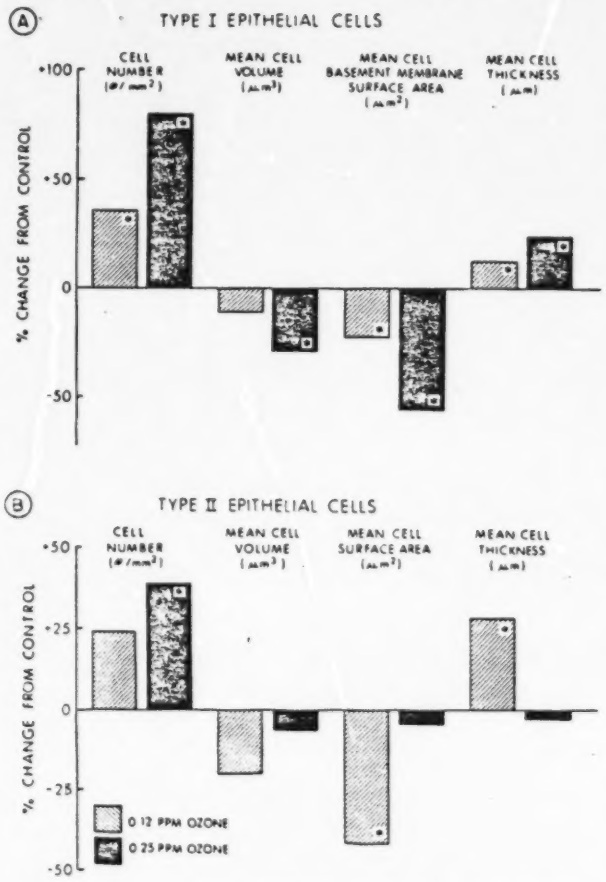

FIG. 8. Epithelium in the proximal alveolar region. The changes in the morphometric characteristics of type 1 and type 2 epithelial cells expressed as percent change from control values following exposure to 0.12 and 0.25 ppm O_3 for 6 weeks. * $p < 0.05$ for analysis of raw data as reported on Tables 1 and 2.

from animals exposed to 0.12 ppm O_3 and 13/32 sites from animals exposed to 0.25 ppm O_3 were above this level. This suggests that there is a response of the type 1 epithelium in virtually all of the regions throughout the lungs of exposed animals. Since the variability among sites is greater for the animals exposed to 0.25 ppm O_3, it also suggests that there is some variability in the extent of reaction in specific proximal alveolar regions. Because O_3 is a reactive gas, its concentration will decrease as it moves down the airways. It is possible that the extent of tissue reaction to O_3 is determined in part by the path length from the trachea to each specific proximal alveolar region. The statistical analysis of these data as reported in Table 1 was done on the animal means since the statistical significance tests of gas and age effects employ "animal" as the experimental unit.

DISCUSSION

Morphometric techniques were developed to quantitate structural changes in the proximal alveolar region of rat lungs caused by concentrations of O_3 which are at and near the current National Ambient Air Quality Standard of 0.12 ppm and which are encountered in the ambient air of some urban environments. The proximal alveolar region, which in this study is defined as those alveoli adjacent to the first alveolar duct bifurcation, was selectively studied since this is one of the primary sites of O_3-induced lung injury.

Work done by Bartlett, Faulkner, and Cook (2) has suggested that young animals have increased sensitivity to O_3. Unfortunately, this study was not designed in a fashion that permits this question to be definitively resolved. Neonatal and young animals have been shown to be more responsive than older animals to biochemical changes in the lung in response to O_3 exposure (10, 31). Stephens *et al.* (28, 29) have reported that postnatal rats are resistant to the toxic effects of O_3 and nitrogen dioxide until they are weaned. They identified significant susceptibility after weaning to the effects of these gases on the proximal alveolar region. Enhanced activity and/or responsiveness of endogenous antioxidant systems in rats prior to weaning (36) and/or the effects of change in diet from mother's milk to rat chow were postulated to be potential reasons for resistance of neonatal rats to oxidant-mediated lung injury. The findings of the current study demonstrate that low levels of O_3 do not significantly interfere with completion of alveolarization and lung maturation and that young animals do not show greater changes in lung structure in response to exposure to low levels of this oxidant. Since the juvenile group in the present study was exposed from 1 day to 6 weeks of age, the morphometric and morphologic changes which were found may have primarily occurred after weaning at 3 weeks of age and resulted in overall changes which were not substantially different from those occurring in the adult rats.

The changes which were morphometrically defined in type 1 cells in the proximal alveolar region of rats exposed to both 0.12 and 0.25 ppm O_3 suggest that an increased portion of this population of epithelial cells is at an intermediate stage of cell differentiation between type 1 and type 2 cells. The numbers of type 1 cells were significantly increased, they were smaller in volume, they covered less surface area, and they were thicker than those of control animals. The presence of epithelial cells which were intermediate in appearance between type 1 and type 2 cells have been reported by other investigators (3, 9, 22). The combined findings of qualitative evidence of type 1 cell injury, increased numbers of type 2 cells, and morphometrically documented changes in characteristics of type 1 and type 2 cells indicate that inhalation of 0.12 or 0.25 ppm of O_3 causes injury to the epithelium and an increase in the rate of epithelial cell proliferation compared to controls.

The epithelial cell alterations caused by O_3 reported in the present study are supported by previous autoradiographic, histochemical, and morphometric studies of the proximal alveolar region. Evans *et al.* (12, 13) reported that there was increased mitotic activity by type 2 cells in the proximal alveolar region of rats exposed to less than 1 ppm O_3. Similar increases in type 2 cell mitoses were seen in the respiratory bronchioles of bonnet monkeys exposed to O_3 (11). Histochemical studies by Castleman, Dungworth, and Tyler (5, 6) showed that

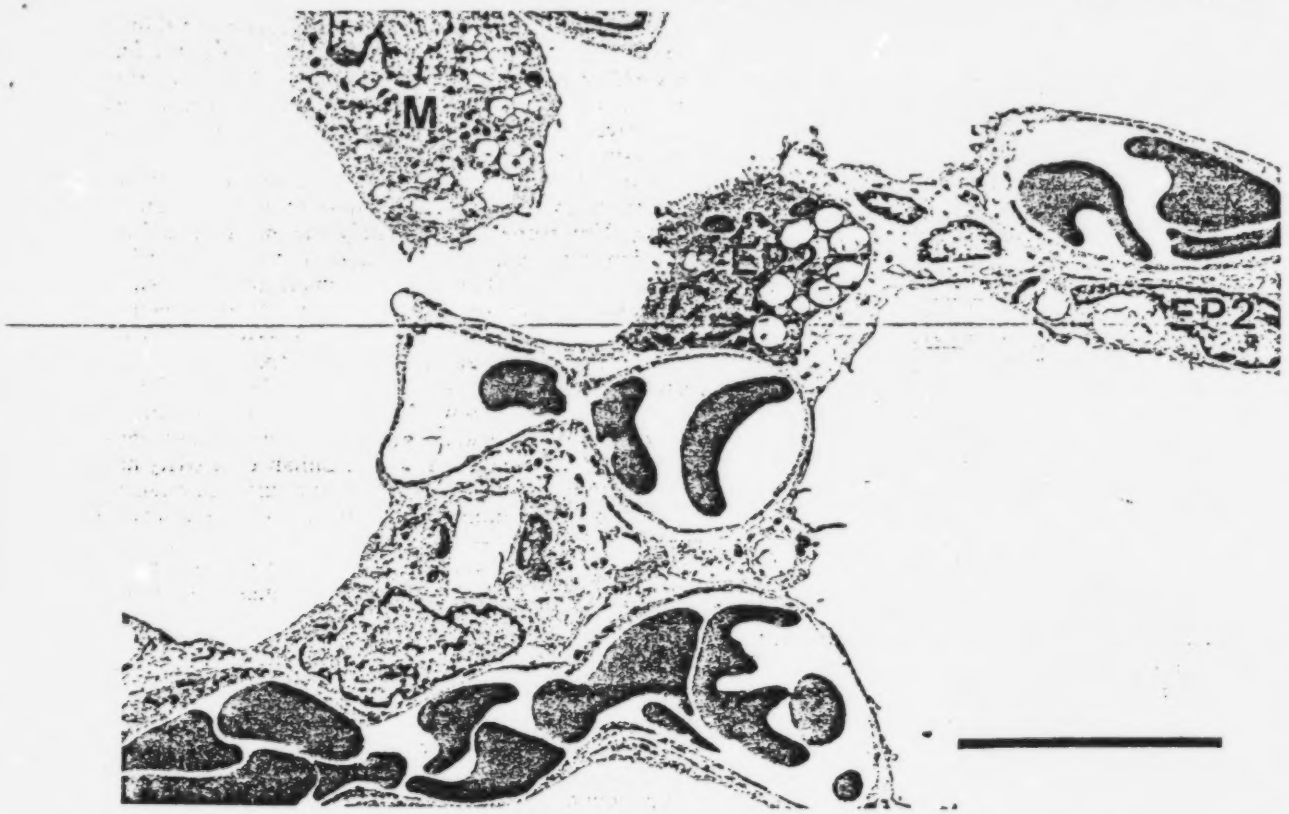

FIG. 9. Alveolar septum from an adult rat exposed to 0.12 ppm O_3 for 6 weeks. Two type 2 epithelial cell profiles (*EP2*) are in close proximity, which reflects the increased number of these cells in this region after O_3 exposure. An alveolar macrophage (*M*) is nearby. Bar = 10 μm.

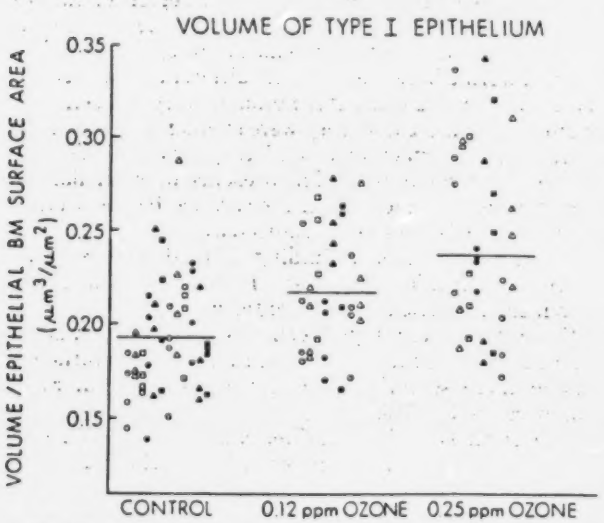

FIG. 10. Volume density of type 1 epithelium in the proximal alveolar regions of control and O_3-exposed rats. Each column of four identical symbols represents the four data values calculated for this parameter for each animal. The *horizontal bar* in each symbol cluster represents the mean for the animals in that group. This figure illustrates the linear relationship between O_3 concentration and increases in the volume density of type 1 epithelium after 6 weeks of exposure.

increased amounts of acid phosphatase and β-glucuronidase were present in the thickened epithelium of the terminal bronchioles and adjacent alveoli of O_3-exposed rats. They suggested that O_3 may have damaged the plasma membrane of lysosomes which contain the enzyme and allowed leakage into the cytoplasm. In a morphometric study of the proximal alveolar region of rats, Boorman *et al.* (3) reported that after 90 days exposure to 0.2 ppm O_3 there were increases in the arithmetic mean thickness of the air-blood barrier and in the volume density of the epithelium, especially type 2 cells. Biochemical studies of rat lung tissue after O_3 inhalation revealed increases in several enzymes, including succinate oxidase, reduced nicotinamide adenine dinucleotide phosphate-cytochrome *c* reductase, and glucose-6-phosphate dehydrogenase, indicating alterations in lung metabolism (23). These data and those in the current study are consistent with the proposal by Eustis *et al.* (11) that O_3 inhalation produces a new level of equilibrium between cell loss and replacement in the respiratory epithlium. Whether or not this type of response of the alveolar epithlium leads to irreversible disease will have to await results of further lifetime exposure studies since the long-term effects of low levels of O_3 are unknown.

Ozone inhalation caused a significant alteration in the

interstitium of both age groups of rats exposed to 0.25 ppm O_3 but did not cause changes in rats exposed to 0.12 ppm O_3. The increase in interstitial matrix volume correlated with qualitative observations of areas of increased collagen in the proximal alveolar region (Fig. 5). An increase in the total number of interstitial macrophages was found in the adult animals (Fig. 7). These findings suggest that there was a persistent inflammatory response in the interstitium caused by inhalation of 0.25 ppm O_3. Previous studies of rats exposed to higher concentrations of O_3 in the range of 0.5 to 2 ppm have demonstrated evidence of increased collagen synthesis (19, 21) and microscopic evidence of fibrosis (3, 14, 21). Inhalation of 1.5 ppm O_3 has been used as a model for pulmonary fibrosis in rats (18). The increase in noncellular interstitium reported in the present study is probably associated with a small, but significant fibrotic reaction.

The proximal alveolar region has significant functional importance because it is the critical intersection between the conductive and respiratory portions of the lung. This area is a focal point of several pathologic lesions, including centrilobular emphysema, asbestos fiber deposition (4), and injury caused by oxidant gases such as O_3 and nitrogen dioxide. Loss of alveolar tissue, as seen in centrilobular emphysema, or fibrotic changes caused by inhalation of particulates and oxidant gases can affect normal gas exchange in this region. These changes may also alter the normal distribution of air to more distal portions of the lung parenchyma. The approach to tissue isolation and morphometric analysis presented here provide a sensitive quantitative method to evaluate pathologic changes in the proximal alveolar region of the lung caused by injurious elements. The results demonstrate that at low levels of O_3 there is a relatively linear relationship between cell structural alterations in the proximal alveolar tissue and the concentration of O_3 used in the exposure.

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